Original Paper

Digestion

Digestion 2009;79:99–108
DOI: 10.1159/000209219

Received: December 9, 2008
Accepted: January 22, 2009
Published online: March 18, 2009

Early *Helicobacter pylori* Eradication Restores Sonic Hedgehog Expression in the Gastric Mucosa of Mongolian Gerbils

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Key Words
Helicobacter pylori · Eradication · Sonic hedgehog

Abstract
Background and Aim: Sonic hedgehog (Shh) is a morphogen involved in the homeostasis of the gastric fundic glands. Alterations of gastric mucosal Shh expression after eradication of *Helicobacter pylori* were examined. ***Method:*** Mongolian gerbils were inoculated with *H. pylori* at the age of 5 weeks. *H. pylori* eradication was then carried out at 12, 24 or 48 weeks after the inoculation, and the gerbils were examined at 10 weeks after the eradication. Gastric inflammation was evaluated by the tissue myeloperoxidase activity and the histological scoring. Immunohistochemistry and in situ hybridization were performed for determining the Shh expression. ***Results:*** Significant decrease of the myeloperoxidase activity and scores for acute and chronic inflammation as well as atrophy were observed in the *H. pylori*-eradicated gerbils as compared with the findings in the non-*H. pylori*-eradicated gerbils. Significant increase of the horizontal length of the area positive for Shh expression was noted in the *H. pylori*-eradicated gerbils as compared with that in the non-*H. pylori*-eradicated gerbils. Earlier eradication promoted better restoration of Shh expression. 50% of the animals of the 24-week eradication group and all animals in the 48-week eradication group exhibited heterotopic proliferative glands. In the animals showing heterotopic proliferative glands, the front line of Shh regeneration was cut off at the point of development of heterotopic proliferative glands. ***Conclusion:*** *H. pylori*-associated deregulation of Shh expression that could be linked to gastric atrophy and the associated preneoplastic transformation appears to be reversible with early *H. pylori* eradication.

Introduction

Epithelial cells in the gastrointestinal tract mucosa are continuously replaced from a pool of precursor cells. After the specialization and maturation, these cells fulfill a variety of functions, including those related to mucosal defenses against invasion by pathogens, digestion and absorption of food, and regulation of intermediary metabolism. The cell fate of the differentiating epithelial cells is coupled to positional information that is received as they migrate along the axis of renewal [1]. In recent years, it has become increasingly clear that such information is provided by gradients of morphogens [2]. Morphogens are secreted soluble molecules with dose-dependent effects on the cellular fate, and belong to a small number of families with a common signaling receptor [3].

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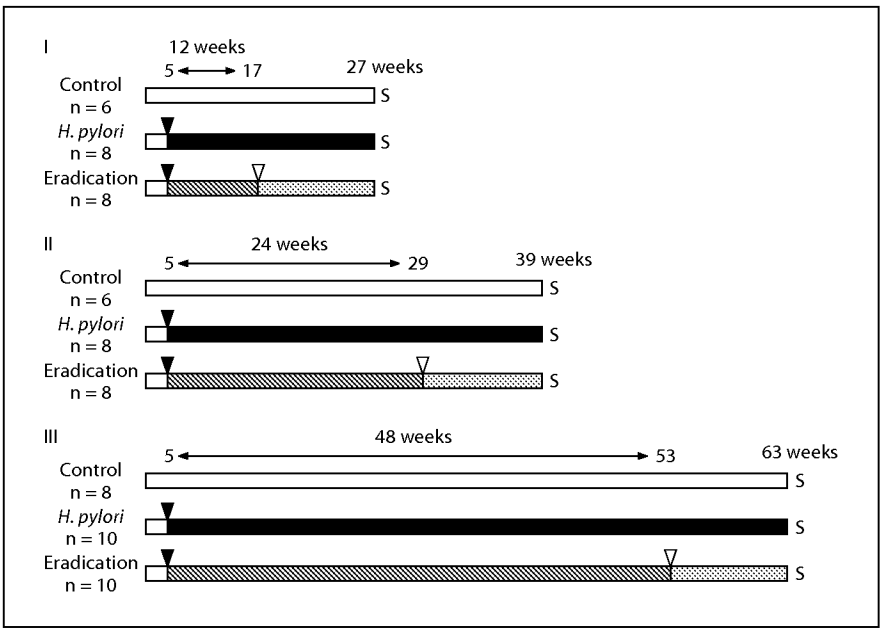

Fig. 1. Experimental design. Mongolian gerbils were inoculated with *H. pylori* at the age of 5 weeks. *H. pylori* eradication was carried out at 12, 24 or 48 weeks after the inoculation. Gerbils were examined at 10 weeks after the eradication. ▼ = *H. pylori* inoculation; ▽ = eradication; S = sacrifice.

Members of the hedgehog family of morphogens play an important role in the development and homeostasis of the gastrointestinal mucosa. Mammals have three hedgehog genes: sonic hedgehog (Shh), Indian hedgehog, and desert hedgehog. The gastric mucosa of Shh-null mice has been reported to show epithelial hyperplasia and express alkaline phosphatase, a sign of intestinal-type differentiation [4]. Shh has been shown to be abundantly expressed in the fundic glands of the adult stomach and to be involved in fundic gland homeostasis [5]. Shh protein is expressed not only in the parietal cells, but also the zymogenic cells and mucous neck cells of the fundic glands in Mongolian gerbils [6]. The expression level of Shh appears to be closely correlated with fundic gland differentiation; the expression has been shown to be lost in intestinal metaplasia of the stomach and increased in ectopic fundic glands, such as in gastric metaplasia of the esophagus and Meckel's diverticulum [7].

Infection of the stomach by *Helicobacter pylori (H. pylori)* may lead to the development of gastric atrophy, peptic ulcer disease, gastric MALT lymphoma and gastric cancer [8]. We have previously shown that disturbances of epithelial cell differentiation induced by *H. pylori*-associated gastritis in Mongolian gerbils are correlated closely with changes in the Shh expression [6]. Loss of Shh has recently been reported to be an early change in carcinogenesis of the gastric mucosa, prior to neoplastic transformation [9]. Furthermore, we reported that the suppressed Shh expression in the gastric mucosa by *H. pylori* infection was significantly restored following *H. pylori* eradication in humans [10].

The aim of the present study was to assess the alteration of the gastric mucosal Shh expression after eradication of *H. pylori* in an animal model.

Materials and Methods

Mongolian Gerbil Model of H. pylori *Infection*

The animals were allocated to experiments I, II or III (fig. 1). In experiment I, 22 male Mongolian gerbils (MGS/Sea, 5 weeks old; Kyudo Co., Ltd., Fukuoka, Japan) were divided into three groups: *H. pylori*-infected group (n = 8), *H. pylori*-eradicated group (n = 8), and control group (n = 6). After overnight food deprivation, the first two groups were administered suspensions of *H. pylori* (ATCC43504: 10^8 colony-forming units (CFU)/ml, 15 ml/kg) at the age of 5 weeks, while the control group (n = 6) was administered buffer solution alone [11]. All the animals were allowed free access to water and a standard pellet diet (CE-2; Clea Japan, Tokyo, Japan). As *H. pylori* eradication treatment in the *H. pylori*-eradicated group, triple therapy with lansoprazole (10 mg/kg), amoxicillin (3 mg/kg), clarithromycin (30 mg/kg) (in 0.5% wt/wt carboxymethyl cellulose sodium salt) was administered intragastrically twice daily for 2 successive days at 12 weeks after the *H. pylori* inoculation, while gerbils in the control group and *H. pylori*-infected group were administered buffer solution alone. Ten weeks after the eradication, the gerbils were examined under ether anesthesia after 16 h food deprivation and finally sacrificed with an overdose of ether.

In experiment II, eradication treatment was administered at 24 weeks after the inoculation in the *H. pylori*-eradicated group, while the control group and *H. pylori*-infected group were administered buffer solution alone. The gerbils were examined 10 weeks after the eradication.

In experiment III, 28 gerbils were divided into three groups: *H. pylori*-infected group (n = 10), *H. pylori*-eradicated group (n = 10), and control group (n = 8). Eradication treatment was administered at 48 weeks after the inoculation in the *H. pylori*-eradicated group, while the control group and *H. pylori*-infected group were administered buffer solution alone. The gerbils were examined 10 weeks after the eradication.

All experiments and procedures carried out on the animals were approved by the Keio University Animal Research Committee (No. 056188).

Confirmation of H. pylori *Infection*

Eight weeks after the bacterial inoculation, blood samples were taken from an orbital vessel and the serum level of anti-*H. pylori* IgG was measured using an enzyme-immunoassay (EIA) kit (Determiner *Helicobacter pylori* antibody kit, Kyowa Medex Co., Tokyo, Japan) modified by changing the original secondary antibody to peroxidase-labeled rabbit anti-mouse IgG (Dako Japan, Kyoto, Japan). Gerbils producing samples with an optical density on anti-*H. pylori* IgG EIA greater than 0.1 at this period were recognized as *H. pylori*-colonized cohorts [12].

H. pylori infection and its eradication status were confirmed by determining the number of CFUs in a microaerobic bacterial culture. Briefly, diluted homogenates of the stomachs were plated on to *Brucella* agar plates containing 10% horse blood, 2.5 μg/ml amphotericin B, 9 μg/ml vancomycin, 0.32 μg/ml polymyxin B, 5 μg/ml trimethoprim, and 50 μg/ml 2,3,5-triphenyltetrazolium chloride. The plates were then incubated at 37°C in a microaerobic atmosphere for 7 days [13].

Measurement of Myeloperoxidase Activity

Immediately after the mice were sacrificed, the stomachs of the animals were removed and opened along the greater curvature. Tissue samples of the gastric mucosa were collected in tubes containing PBS and protease inhibitors (100 μmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) and sonicated on ice in 30 consecutive 0.5-s bursts at 0.5-s intervals, at a power setting of 150 W (VCX 50; Sonics & Materials, Inc., Newton, Conn., USA). The total protein content in the homogenates was measured by modified Lowry's method, as described by Smith et al. [14].

Myeloperoxidase activity, as an index of tissue-associated neutrophil accumulation, was determined by the method described by Grisham et al. [15], with some modification. Aliquots containing 100 μl of the mucosal homogenates were centrifuged at 8,000 rpm for 15 min at 4°C to separate the pellets of insoluble cellular debris. The pellets were rehomogenized in an equal volume of 0.05 mol/l of potassium phosphate buffer (pH 5.4) containing 0.5%-hexadecyltrimethylammonium bromide. The samples were then centrifuged at 8,000 rpm for 15 min at 4°C and the supernatants were stored. The myeloperoxidase activity in the supernatants was estimated by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of myeloperoxidase causing a change of absorbance at 655 nm of 1.0/min at 25°C.

Histopathological Grading

The stomach tissue specimens were stained with hematoxylin and eosin. The following four histological parameters were graded from 0 (absent/normal) to 3 (maximal intensity) on a visual analogue scale: activity of gastritis (granulocytic infiltration), severity of inflammation (lymphocytic and plasma cell infiltration), glandular atrophy, and intestinal metaplasia according to the updated Sydney classification [16]. The histopathological scores were evaluated at the mid-portion of the specimens.

Specimens for histology were assessed by a single investigator who was blinded to the *H. pylori* infection status.

Immunohistochemistry

The antibody used for the immunohistochemistry was a goat polyclonal anti-Shh antibody (N-19, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) directed against the Shh precursor protein [6]. The stomach tissue specimens were fixed in 10% neutralized buffered formalin, processed by the routine method, and embedded in paraffin. The paraffin sections were placed on poly-L-lysine-pretreated slides, dewaxed and then rehydrated. Antigen retrieval for the Shh antibody was performed in boiled citrate buffer solution (97°C, 5 min, 10 mM, pH 6.0). After the antigen retrieval, endogenous peroxidase activity was quenched by treatment with 0.3% hydrogen peroxide for 15 min. The sections were rinsed and non-specific protein binding was blocked with a blocking reagent (Protein Block Serum-Free, Dako Cytomation). Sections were incubated overnight with primary antibody at 4°C. After washing with Tris-buffered saline with Tween 20 (TBS-T), the slides were incubated with horseradish peroxidase (HRP)-labeled anti-goat IgG (Histofine, Simple stain MAX-PO, Nichirei, Tokyo, Japan) for 30 min at room temperature. Thereafter, the color was developed with 3,3′-diaminobenzidine tetrahydrochloride solution. Counterstaining was performed with Gill's hematoxylin. The horizontal length (mm) of the region showing Shh expression was measured [6].

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Taqman quantitative real-time RT-PCR was performed to detect Shh mRNA and GAPDH mRNA from the total RNA isolated from the anterior wall of the stomach with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The following primers were used to amplify the Shh mRNA: Shh-F (5′-CCCGGTCAGATGTGGTGATG-3′), Shh-R (5′-TGAGGATGGCCACCATTCA-3′), and Shh-Taq (5′-FAM-CTGGGCCCTCGTAGTGCAGAGACTCCT-TAMARA-3′); in addition, the following primers were used to amplify GAPDH mRNA as the internal control: GAPDH-F (5′-TTCAACGGCACAGTCAAGGC-3′), GAPDH-R (5′-GCCTTCTCCATGGTGGTGAAG-3′), and GAPDH-Taq (5′-FAM-CCCATCACCATCTTCCAGGAGCGAGA-TAMARA-3′). The Shh mRNA expression levels were normalized to the GAPDH mRNA expression levels [6, 17].

In situ Hybridization

Abdominal tissue specimens from the gerbils were fixed overnight in 4% paraformaldehyde, processed by the routine method, and embedded in paraffin. Paraffin sections (4–6 μm in thickness) were used for the in situ hybridization using a digoxigenin-labelled in situ Shh probe, in accordance with a previously published method [6].

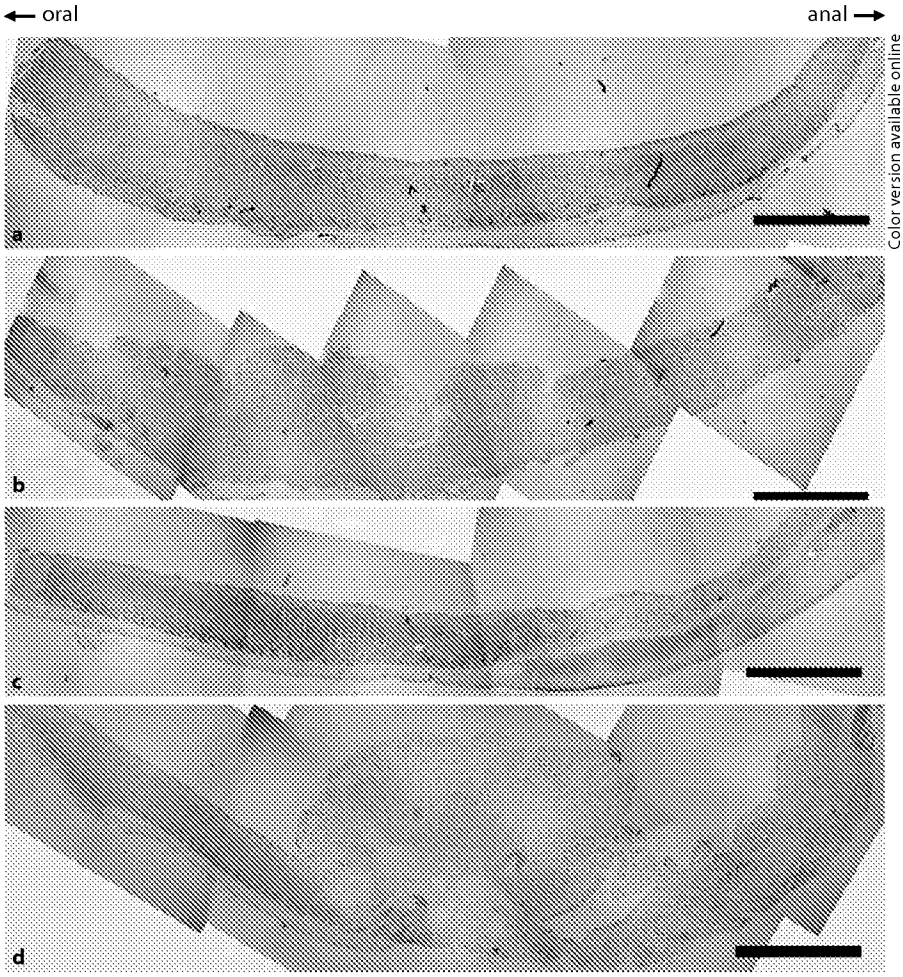

Fig. 2. Shh immunohistochemistry in experiment II. Several high-resolution micrographs from the oral (left) to the anal (right) side of the gastric mucosa are superimposed. About 50% of the animals in the group administered eradication therapy at 24 weeks exhibited heterotopic proliferative glands. In the cohort with heterotopic proliferative, the front line of Shh regeneration was cut off at the point of appearance of the heterotopic proliferative glands. **a** Control. **b** *H. pylori*-infected gerbil. **c** Gerbil from the 24-week eradication group not showing heterotopic proliferative glands. **d** Gerbil from the 24-week eradication group showing heterotopic proliferative glands. Bar = 2 mm.

Statistical Analysis

All data were expressed as mean ± SD; $p < 0.05$ was considered to denote statistical significance. The data were analyzed by one-way analysis of variance, followed by Scheffé's multiple comparisons.

Results

All gerbils of the *H. pylori*-infected group were confirmed as *H. pylori*-positive, and all gerbils of the control and *H. pylori*-eradicated groups were confirmed as *H. pylori*-negative in the present study.

About 50% of the animals in the group administered eradication therapy at 24 weeks and all the animals in the group administered eradication therapy at 48 weeks exhibited heterotopic proliferative glands, which was represented by hyperplastic changes with multifocal cystic dilatation, infiltration of inflammatory cells in the lower gastric body [18]. In the cohort with heterotopic proliferative glands, the front line of Shh regeneration was cut off at the point of appearance of the heterotopic proliferative glands (fig. 2).

In situ hybridization shows that Shh mRNA expression was clearly localized with a gradient in the fundic gland region of the control group and the 48-week eradication group (fig. 3). Shh mRNA expression was restored in the *H. pylori*-eradicated gerbils as compared with that in the *H. pylori* positive gerbils.

Significant decrease of the myeloperoxidase activity and histological scores for acute and chronic inflammation was observed in the *H. pylori*-eradicated gerbils as compared with that in the *H. pylori*-positive gerbils (fig. 4).

Significant increase of the horizontal extent of the area positive for Shh expression was observed in the *H. pylori*-eradicated gerbils compared with that in the *H. pylori*-

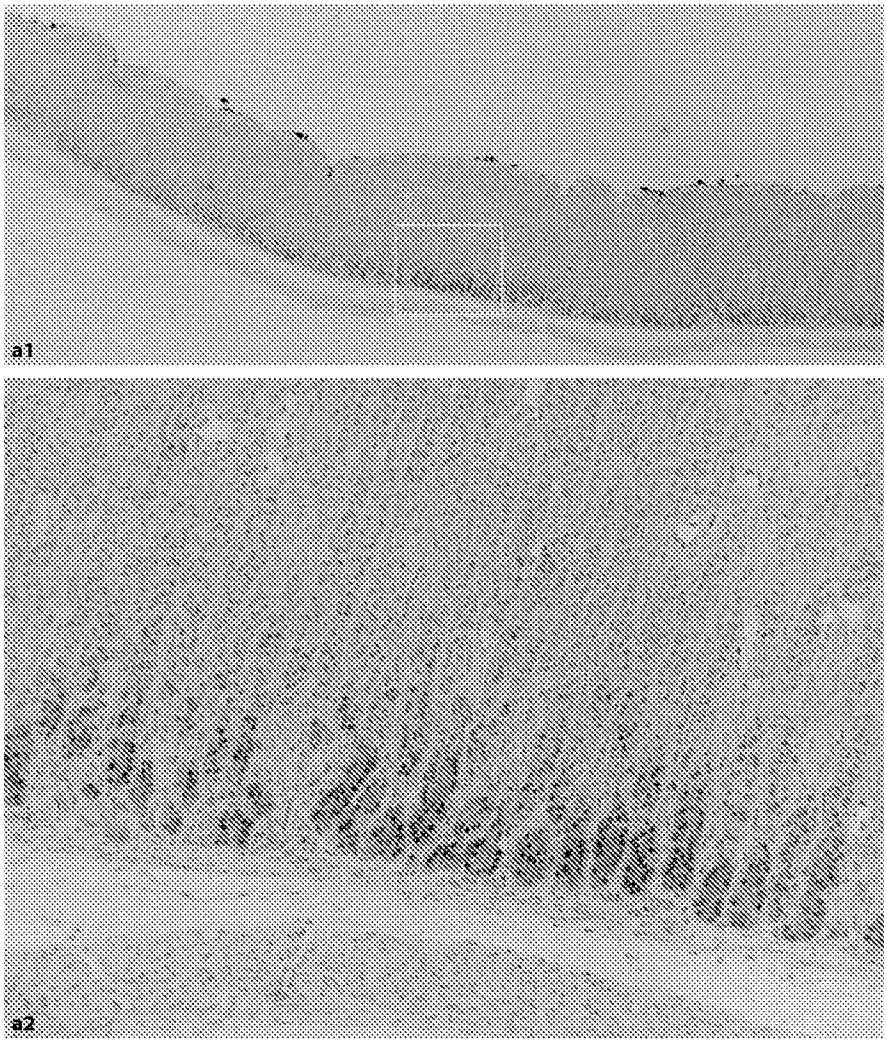

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Fig. 3. Shh in situ hybridization in experiment III. Shh mRNA expression was clearly localized with a gradient in the fundic gland region of the control group. **a1** Control (×16). **a2** Magnification of boxed area in **a1** (×100).

positive gerbils (eradication at 12, 24 weeks: $p < 0.001$, eradication at 48 weeks: $p < 0.01$). The earlier the eradication, the better the restoration of Shh expression.

Significant increase of the Shh mRNA expression levels were observed in the gerbils of the 12- and 24-week eradication groups as compared with that in the *H. pylori*-positive gerbils ($p < 0.05$) (fig. 5).

Significant decrease of the mucosal atrophy scores based on the updated Sydney system was observed in the *H. pylori*-eradicated groups (eradication at 12, 24 weeks: $p < 0.001$, eradication at 48 weeks: $p < 0.05$) (fig. 6). Earlier eradication promoted greater improvement of the gastric atrophy.

Discussion

H. pylori infection causes a chronic inflammatory response in the human host, associated with histological changes such as glandular atrophy and intestinal metaplasia [10]. Morphogens are soluble signals that are secreted in concentration gradients through a tissue and regulate epithelial cell differentiation. We have previously reported that *H. pylori* infection suppresses the expression of a gastric morphogen in Mongolian gerbils [6]. In the present study, we demonstrated that the suppressed Shh expression in the gastric mucosa by *H. pylori* infection was restored after eradication of the bacteria, and that earlier eradication promoted better restoration of the Shh expression in the gastric mucosa of the Mongolian gerbil.

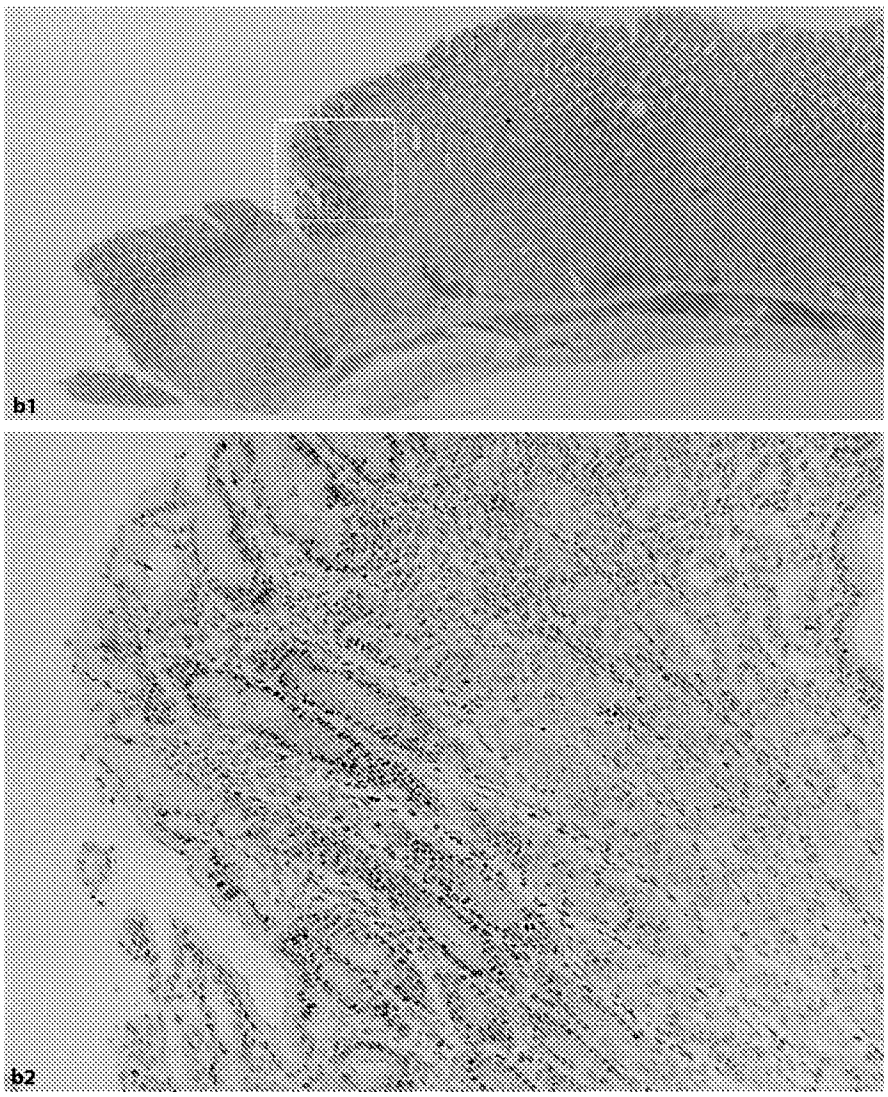

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Fig. 3. Shh in situ hybridization in experiment III. Most of the Shh mRNA expression has disappeared in the fundic gland region of the *H. pylori*-infected group. Only a faint positivity has been detected in a localized surface mucosa. **b1** *H. pylori*-infected gerbil (×16). **b2** Magnification of boxed area in **b1** (×100).

H. pylori eradication therapy has been shown to result in the reduction and eventual disappearance of inflammation [19, 20]; however, evidence of its effect on the mucosal atrophy is still conflicting. While some studies have shown beneficial effects of *H. pylori* eradication on the gastric histology [21–24], others have observed no significant improvement at all [25, 26]. In the present study, significant improvement of the myeloperoxidase activity and acute inflammation and atrophy scores was observed in the gastric mucosa of the *H. pylori*-eradicated group of gerbils. In addition, the Shh expression levels in the gastric mucosa were also significantly restored in this group of animals. These results suggest that *H. pylori*-associated deregulation of Shh expression that could be linked to gastric atrophy is reversible after *H. pylori* eradication, especially in the early phase.

H. pylori infection, atrophic gastritis and intestinal metaplasia are well-known risk factors for gastric cancer [27, 28]. Eradication of *H. pylori* has been shown to possibly reduce the risk of gastric cancer in some groups [22, 29]. We have reported that Shh expression has been restored in the human gastric mucosa after *H. pylori* eradication [10]. Loss of Shh has been reported to be an early change of carcinogenesis that occurs in the gastric mucosa prior to neoplastic transformation [9]. Shiotani et al. [30] has indicated that loss of Shh in *H. pylori*-associated atrophic gastritis responds to eradication and the reversibility depends upon the severity of atrophic gas-

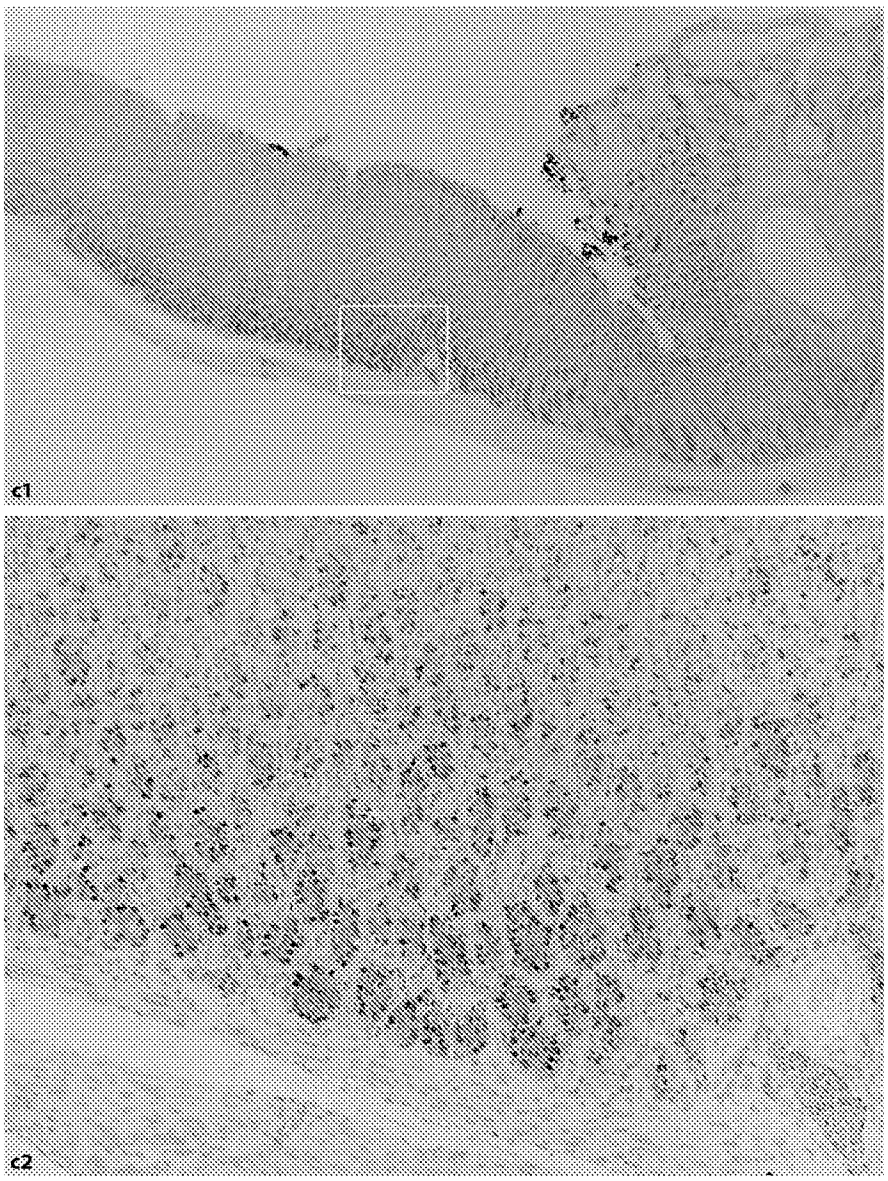

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Fig. 3. Shh in situ hybridization in experiment III. Shh mRNA expression was clearly localized with a gradient in the fundic gland region of the 48-week eradication group. **c1** Gerbil from the 48-week eradication group (×16). **c2** Magnification of boxed area in **c1** (×100).

tritis and the presence of incomplete intestinal metaplasia prior to *H. pylori* eradication. Our findings support the previous clinical study and confirm reversal of gastric preneoplastic transformation following *H. pylori* eradication.

According to Ikeno et al. [31] who reported on the pathological findings in the stomach of Mongolian gerbils at 2, 4, 8, 12, 26, 38 and 52 weeks after *H. pylori* inoculation, histological inflammatory changes reached their peak at 8 and 12 weeks after the inoculation. After 26 weeks of infection, intestinal metaplasia appeared, which increased steadily in extent up to 52 weeks. Glands in the stomach of *H. pylori*-infected gerbils start to proliferate into the submucosa, disrupting the laminal muscularis mucosa. The resultant lesions, termed heterotopic proliferative glands, frequently develop in association with *H. pylori* infection in the glandular stomach of infected gerbils, with minimal dysplastic changes of the constituent cells. Heterotopic proliferative glands often resemble differentiated or mucinous adenocarcinoma, but do not appear to be malignant. Their characteristics: (1) organized polarity of the component cells; (2) differ-

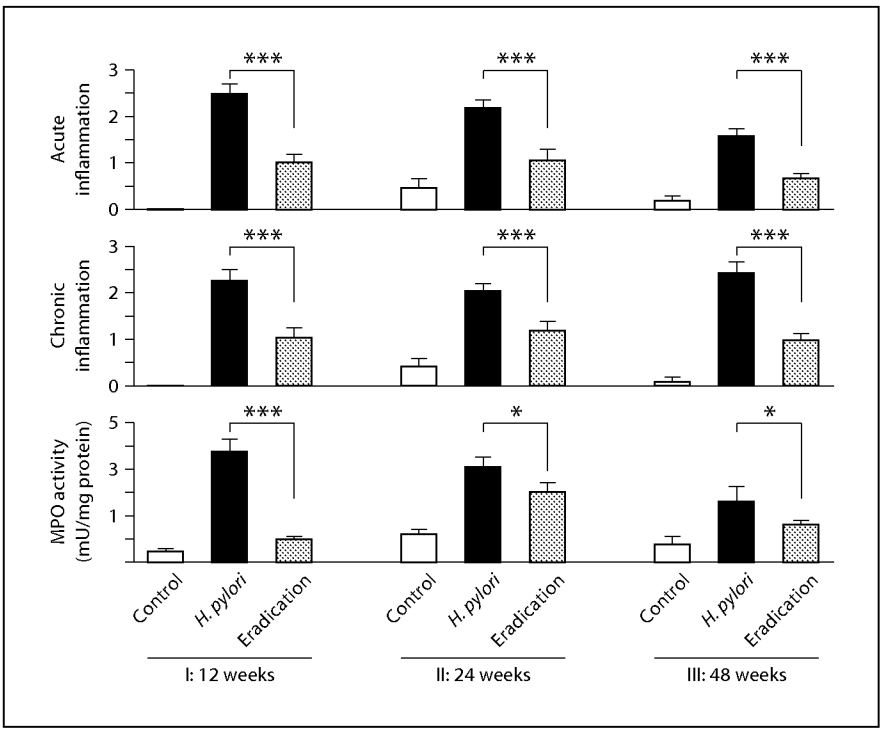

Fig. 4. Acute and chronic inflammation scores and myeloperoxidase (MPO) activity in the stomach. *** $p < 0.001$, * $p < 0.05$ vs. the *H. pylori*-infected gerbils.

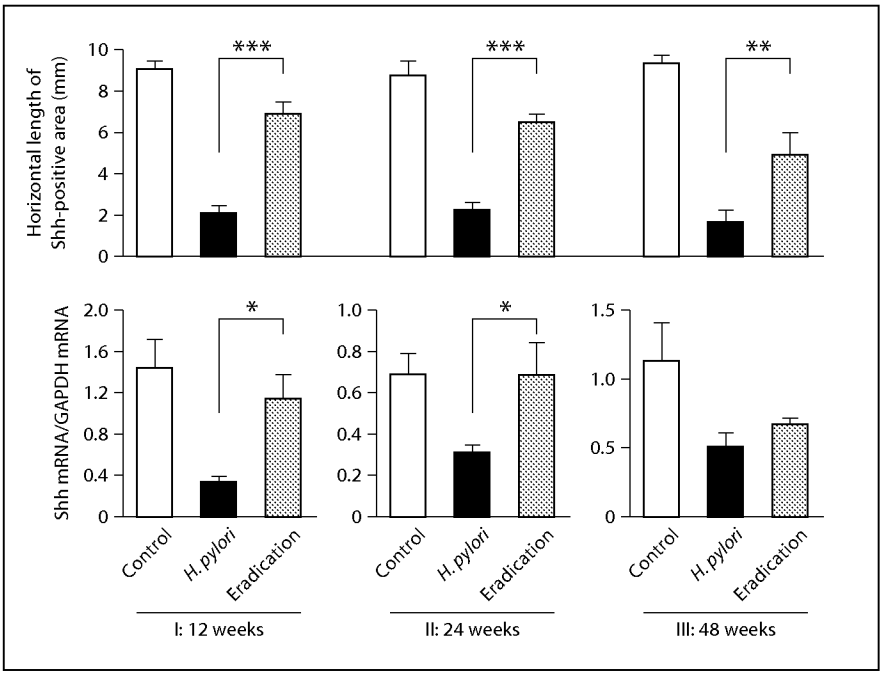

Fig. 5. Horizontal length of the areas showing Shh expression and Shh mRNA expression in the stomach. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ vs. the *H. pylori*-infected gerbils.

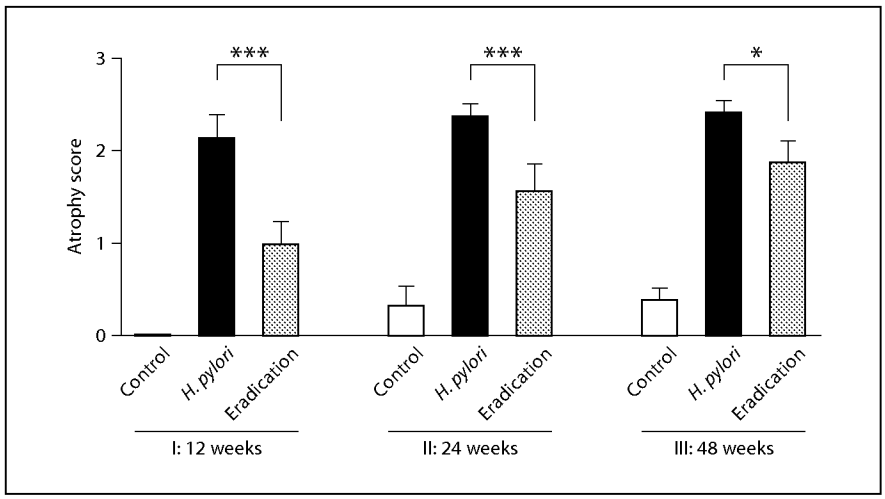

Fig. 6. Atrophy score in the stomach. *** $p < 0.001$, * $p < 0.05$ vs. the *H. pylori*-infected gerbils.

entiation from the gastric-phenotype heterotopic proliferative glands into intestinal-phenotype glands with mature Paneth cells; (3) formation of large cystic dilatations containing mucin, often with calcification; (4) shedding of epithelial cells and necrosis at the tips of the inflammatory cells; (5) organized polarity of proliferating cells exhibiting hyperplastic changes and variable degrees of multifocal cystic dilatations [32]. Cao et al. [33] reported that eradication of *H. pylori* induced apoptosis and suppressed proliferation of heterotopic proliferative glands in the gastric mucosa of the infected gerbils. Furthermore, significant reductions in the area of the heterotopic proliferative glands were observed at 8 and 25 weeks post-eradication [33]. Reversible heterotopic proliferative glands appear to be more related to severe gastritis than being malignant in character. In the present study, 50% of the animals in the group administered eradication therapy at 24 weeks and all the animals in the group administered eradication therapy at 48 weeks exhibited heterotopic proliferative glands. Administration of eradication therapy earlier in the development of heterotopic proliferative glands would be desirable for complete resolution of the gastritis.

Significant improvement of the Shh mRNA expression levels was not observed in the gerbils of the 48-week eradication group as compared with that in the *H. pylori*-positive gerbils. Shh mRNA expression levels were normalized to the GAPDH mRNA expression levels, while the horizontal length of the region showing Shh expression was measured as absolute value. All gerbils in the group administered eradication therapy at 48 weeks exhibited heterotopic proliferative glands which had the hyperplastic change, so we thought that the expression of GAPDH mRNA which standardized Shh mRNA expression levels increased and the significant improvement was not detected as normalized Shh mRNA expression levels.

In conclusion, significant restoration of Shh expression and significant improvement of the gastric atrophy were observed after *H. pylori* eradication therapy. These results suggest that *H. pylori*-associated deregulation of Shh expression that could be linked to gastric atrophy and the associated preneoplastic transformation is reversible after *H. pylori* eradication, especially in the earlier phase of the infection.

Acknowledgements

This study was supported by a Grant-in-Aid for Exploratory Research from the Japan Society for the Promotion of Science (JSPS) (No. 19659057 to H.S.) and a Keio Gijyuku Academic Development Fund. The preliminary results of this communication were presented at the 1st Meeting of Inflammation Conference in Alimentary Tract (ICAT) held in Tokyo, September 16, 2006, and awarded 1st ICAT award.

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